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Nitrogen and Carbon Flows Estimated by ^{15}N and ^{13}C Pulse-Chase Labeling during Regrowth of Alfalfa

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The flow of ^{15}N and ^{13}C from storage compounds in organs remaining after defoliation (sources) to regrowing tissue (sinks), and ^{13}C losses through root or shoot respiration were assessed by pulse-chase labeling during regrowth of alfalfa (*Medicago sativa* L.) following shoot removal. A total of 73% of labeled C and 34% of labeled N were mobilized in source organs within 30 d. Although all of the ^{15}N from source organs was recovered in the regrowing tissue, much of the ^{13}C was lost, mainly as CO_2 respired from the root (61%) or shoot (8%), and was found to a lesser extent in sink tissue (5%). After 3, 10, or 30 d of regrowth, 87, 66, and 52% of shoot N, respectively, was derived from source tissue storage compounds; the rest resulted from translocation of fixed N_2. Overall results suggest that most shoot C was linked to photosynthetic activity rather than being derived from mobilization of stored C in source organs. Furthermore, isotopic analysis of different chemical fractions of plant tissue suggests that between 14 and 58% of the shoot C derived from source tissues was linked to the mobilization of N compounds, not carbohydrates.

Shoot removal by either herbivory or mechanical harvesting causes large modifications at the physiological, biochemical, and molecular level in herbage species (Ourry et al., 1996; Volenec et al., 1996). The importance of organic reserves in the regeneration of new shoots has been recognized for at least 60 years (Grandfield, 1935). The role of carbohydrate storage and mobilization during regrowth has been studied extensively because (a) defoliation removes most of the photosynthetic area; (b) depletion of carbohydrate storage compounds (starch, polyfructans, and soluble carbohydrates) exceeds that of other biochemical pools (proteins, for example); and (c) from a technical point of view, precise molecular storage species and enzyme activities involved in their hydrolysis have been characterized and may be measured easily. Reduced nitrogenase activity in defoliated legumes, for example, has been explained as being due to a shortage of C skeletons in the nodules. However, more recent data show that nitrogenase activity is not strongly coupled to stored C but rather to a modification of O_2 permeability of the nodules that leads to inactivation of nitrogenase (Denison et al., 1992).

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After defoliation of forage legumes, N reserves are required for the initiation of new shoot growth because of the reduction in nitrogenase activity and/or soil N uptake caused by shoot removal (Vance et al., 1979; Groat and Vance, 1981; Kim et al., 1993b). However, the rate of decline of nitrogenase activity may be affected by either the type of germ plasm present in the plant (Vance et al., 1979; Groat and Vance, 1981) or the plant's age (Ta et al., 1990). N reserve mobilization estimated using pulse-chase ^{15}N labeling in alfalfa (*Medicago sativa* L.) (Kim et al., 1991, 1993b) revealed that approximately 40 and 60% of total N in roots and crowns, respectively, was mobilized to supply N to the shoot shortly after defoliation. Between 36 and 46% of the fixed N present in the crowns and roots of effective symbioses 2 d after shoot harvest was translocated into herbage regrowth during the next 35 d (Russelle et al., 1994). Culvenor and Simpson (1991) and Kim et al. (1991) demonstrated that protein N formed the largest pool of stored N in vegetative tissue. Hendershot and Volenec (1993) reported the extensive depletion of specific amino acids and certain buffer-soluble proteins from taproots during shoot regrowth of defoliated alfalfa. These results suggest that mobilization of specific N pools from vegetative tissues to regrowing foliage after cutting is necessary for defoliation tolerance in alfalfa (Volenec et al., 1996). Electrophoretic analysis of soluble proteins of alfalfa taproots showed the presence of three prominent polypeptides of 15, 19, and 32 kD acting as putative vegetative storage proteins (Hendershot and Volenec, 1993). These polypeptides represented 28% of the total soluble proteins extracted from taproots on the day of cutting (Avice et al., 1996). Their mobilization between d 0 and 6 of regrowth was rapid and extensive, and was followed by reaccumulation. Immunolocalization revealed the presence of these vegetative storage proteins in the parenchyma cells of wood rays, and to a lesser extent, in the parenchyma cells of bark alfalfa taproots. At the ultrastructural level these vegetative storage proteins occurred specifically in the vacuoles as an electron-dense, fibrillar material and on the periphery of starch granules (Avice et al., 1996).

Nonnodulated alfalfa plants with similar crown and root dry weights, but with either low starch and high tissue N or high starch and low tissue N, were used to show that maximum herbage yields during regrowth were obtained from plants with high tissue N, despite their low level of

nonstructural carbohydrates (Ourry et al., 1994). Highly significant correlations were also found between the amounts of N available in roots and crown at the beginning of regrowth and (a) the amount of N that was mobilized to new tissues, (b) the amount of N taken up during the regrowth period, and (c) the final shoot yield after 24 d of regrowth. Although the apparent decline in nonstructural carbohydrates is large compared with the N root reserves (Kim et al., 1993b), no correlations between regrowth yield and taproot starch contents were found in plants that varied in their initial root and crown starch contents (Ourry et al., 1994). These researchers suggested that N reserves were used mainly during the first 10 d after defoliation, and that the resulting aerial growth during this period should be sufficient to restore N_2 fixation and/or N uptake to levels similar to those prior to defoliation.

These results are supported by experiments relating root carbohydrate availability to regrowth intensity. Using reciprocally grafted and self-grafted plants of two alfalfa germ plasms selected for contrasting shoot-elongation rates, Fankhauser and Volenec (1989) showed that root-borne factors mediating shoot regrowth and carbohydrate metabolism following herbage removal are less influential than shoot tissue characteristics. In addition, Boyce and Volenec (1992) observed that shoot regrowth was higher in low- than in high-starch plants. These authors suggested that root total nonstructural carbohydrates may not be a major source of energy for shoot regrowth, which does not conflict with the eventual finding that nonstructural carbohydrates in remaining organs of alfalfa exert, directly or indirectly, a strong influence on regrowth capacity (Ta et al., 1990). Boyce and Volenec (1992) hypothesized that the marked decline in root carbohydrates may be due primarily to the use of carbohydrates in root and stubble respiration.

The above-cited literature emphasizes the importance of root N reserves during shoot regrowth following defoliation, and shows that the contribution of C reserves to shoot regrowth must be re-evaluated. The aims of this work using ^{15}N and ^{13}C pulse-chase labeling during regrowth of alfalfa were to (a) quantify the partitioning of ^{13}C flows from storage pools between shoot or root respiration and mobilization to regrowing tissues, and (b) using dual labeling, assess the contribution of N storage compounds (amino acids, proteins) to ^{13}C flows to regrowing shoot and compare this with ^{13}C flows from C compounds *sensu stricto*.

MATERIALS AND METHODS

Plant Material and Culture

Seeds of alfalfa (*Medicago sativa* L. var Europe) were inoculated with a suspension of *Rhizobium meliloti* strain 2011 and germinated on a sand bench. After 15 d, when the primary trifoliate leaves had appeared, seedlings were transplanted in 9-L plastic pots filled with sterile sand, and then irrigated three times per week with a full nutrient solution without N. The nutrient solution contained, in mol m^{-3}, 0.4 KH_2PO_4, 1.0 K_2SO_4, 3.0 $CaCl_2$, 0.5 $MgSO_4$, 0.15 K_2HPO_4, 0.2 Fe-Na EDTA; and, in mmol m^{-3}, 14 H_3BO_3, 5 $MnSO_4$, 3 $ZnSO_4$, 0.7 $CuSO_4$, 0.7 $(NH_4)_6Mo_7O_2$, and 0.1 $CoCl_2$ (Kim et al., 1991). Plants were grown under greenhouse conditions for 3 months with a thermoperiod of 23°C (day) and 18°C (night). Natural light was supplemented for 16 h d^{-1} with fluorescent tubes (Phytor, Claude GTE, Puteaux, France) supplying approximately 150 μmol photons m^{-2} s^{-1} at the canopy height. Plants were then defoliated 6 cm above root level and transferred to a growth cabinet (E15, Conviron, Winnipeg, Canada) modified for $^{13}CO_2$ labeling with a 16-h photoperiod (300 μmol photons m^{-2} s^{-1} at the height of the canopy) and a day/night temperature regime of 23/18°C. Plants were grown hydroponically on a continuously aerated nutrient solution, as previously described by Kim et al. (1991), in a sealed 3.5-L polyvinyl chloride container fitted with a gas-entry hole on the bottom and a gas-exit hole near the top. The nutrient solution was renewed every 5 d to prevent variations in pH and nutrient concentration. Each container held three plants and was covered with modeling clay (Terostat, Frieburg, Germany) to isolate the shoot atmosphere from the root atmosphere. CO_2-free and dehumidified air was bubbled through the nutrient solution throughout labeling and the subsequent pulse-chase periods at a rate of approximately 3 L min^{-1}.

^{15}N and $^{13}CO_2$ Labeling, Pulse-Chase, and Harvests

The $^{13}CO_2$ assimilation and labeling experiments were adapted from procedures previously described by Kouchi et al. (1986), Cliquet et al. (1990), and Schnyder (1992). We have shown previously (Kim et al., 1991; 1993b) that regrowth of alfalfa can be divided into two different periods: one corresponding to depletion of C and N reserves (first 10 d of regrowth), and a second corresponding to an accumulation of storage compounds. To label these compounds uniformly, the $^{13}CO_2$ and ^{15}N labelings commenced on the 20th d after transfer of the plants into the growth cabinet, when rates of reaccumulation of N reserves were higher (Kim et al., 1993a, 1993b), and were continued for 10 d. A constant ^{13}C excess in the atmosphere of the chamber was obtained by continuously mixing a small amount of $^{13}CO_2$ diluted in N_2 (*F*1 bottle: 99 volumes of N_2 and 1 volume of CO_2 with 100% ^{13}C) with industrial $^{12}CO_2$ (*F*2 bottle: 76 volumes of N_2, 19 volumes of O_2, and 5 volumes of CO_2, with a ^{13}C abundance of 1.078%). The flow rate from the *F*1 bottle was metered as 2/100th of the flow rate of the *F*2 bottle by means of two mass-flow controllers (Tylan Corp., Carson, CA). A homemade controller operated the mass controllers from a master box in response to the output from a CO_2 IR analyzer (ADC 7000, ADC, Hoddesdon, UK) to maintain a constant ^{13}C atom % of 1.49 and a constant CO_2 concentration of 450 μL L^{-1} in the chamber. The final ^{13}C level of CO_2 was determined on triplicate samples of the atmosphere taken twice each day (11 AM and 5 PM) through an exit valve using previously evacuated 30-mL flasks. During the 10-d labeling period, plants were grown with a low N concentration (0.2 mM $^{15}NH_4$ $^{15}NO_3$, with 5.00 atom % ^{15}N excess renewed every 5 d) to label the N reserves uniformly without affecting N_2 fixation rates, as previously described (Kim et al., 1993b). It should be stated

that this low N concentration in the solution did not affect nitrogenase activity. This is supported by the fact that the solution was entirely depleted of N after 3 d. Under similar experimental conditions. Kim et al. (1993b) found that N_2 fixation rates were not consistently affected by this low-concentration, intermittent N supply. At the end of $^{13}CO_2$ labeling, the assimilation cabinet was opened and quickly purged with ambient air. The plants were then defoliated 6 cm above the shoot/root junction (d 0) and allowed to regrow for 30 d, with the same day/night temperature and photoperiod regime, without N in the nutrient solution, and with ^{13}C unenriched CO_2 (natural atmosphere with 350 $\mu L\ L^{-1}$ and a natural abundance of 1.108%). The ^{13}C and ^{15}N remaining in the roots or redistributed within the plants were determined 0, 3, 6, 10, 14, and 30 d after defoliation, i.e. after the end of $^{13}CO_2$ assimilation and ^{15}N labeling, by corresponding harvests of triplicate containers (9 plants). Control plants (i.e. nondefoliated) continued to grow in a greenhouse (conditions of photo- and thermoperiod similar to those used in the growth chamber) and were harvested on the same cutting date (d 30) as the defoliated plants. Harvested plants were separated into lateral roots, taproots, leaves, stems remaining in the crown (i.e. below the level of defoliation), and regrowing leaves and stems. Each sample was immediately frozen at −80°C, freeze-dried, weighed, ground, and stored at −30°C for subsequent analysis.

Shoot Respiration Measurements

During labeling and the chase periods, at the beginning and end of each photoperiod, CO_2-free air (obtained from ambient air compressed to approximately 8 MPa by a screw compressor, and passed through a NaOH column) was injected for 20 min into the cabinet to evacuate CO_2 and, thus, to reduce plant reassimilation of $^{13}CO_2$ lost by respiration. During the night period, the growth cabinet had a confined atmosphere and dark respiration of the alfalfa shoot was determined by measurement of accumulated atmospheric CO_2 (CO_2 analyzer model 7000, ADC). The ^{13}C enrichment of dark-respired CO_2 was measured in triplicate samples of the atmosphere taken at the end of the dark period through an exit valve using previously evacuated 30-mL flasks. During the light period, the growth cabinet was ventilated by atmospheric air, so the loss of ^{13}C by light respiration was measured by the determination of the ^{13}C content of CO_2 trapped on a soda lime column positioned in the air exit of the growth chamber. Assuming that the ^{13}C enrichment of respired substrates was similar during the day and night, the total loss of CO_2 during the light period was estimated from total ^{13}C lost during the day and from the ratio of $^{13}C/^{12}C$ in dark-respired CO_2. Although this assumption has not been proven, it is supported by the fact that ^{13}C-labeled substrates correspond to storage compounds rather than to immediately derived substrates from photosynthetic activity.

Root System Respiration Measurements

Respired CO_2 from the root system was collected from each container by trapping in soda lime columns renewed every 24 h and used for C and ^{13}C analysis. The CO_2-free air was bubbled through the solution at a rate of approximately 3 L min^{-1} to ensure a rapid renewal of root atmosphere approximately every 20 s. Samples of the nutrient solution were taken at each renewal time (every 5 d) and analyzed after freeze-drying to measure the amount of dissolved CO_2. We refer to the CO_2 or ^{13}C lost by root system respiration as the amounts found in the gaseous effluent and in the nutrient solution.

Chemical Fractionation and Isotopic Analysis

N, C, ^{13}C, and ^{15}N contents of plant samples, soda lime CO_2 traps, and nutrient solutions were measured in continuous flow using a C/N analyzer linked to an isotope ratio mass spectrometer (Roboprep CN and mass spectrometer, Europa Scientific, Crewe, UK). The atmospheric samples of the growth cabinet were previously purified in a gas analyzer (Roboprep G+, Europa Scientific) before injection into the mass spectrometer.

Each plant sample was then subjected to the following chemical fractionation adapted from Atkins and Canvin (1971) and Gordon et al. (1977): One hundred milligrams of finely ground freeze-dried plant material was mixed in 20 mL of ethanol (80%, v/v) and boiled under reflux for 30 min. The ethanol-soluble fraction was filtered, dried by rotary evaporation under a vacuum, and dissolved in 7.5 mL of water. The residue remaining after centrifugation at 4000*g* for 10 min at 4°C (mainly pigments, waxes, and lipids) was then used for isotopic analysis as described above, whereas the water-soluble fraction was passed through a H^+ column (Dowex 50W, Sigma) and a formate-form Cl^- column (Dowex 1, Sigma) mounted on-line. Soluble sugars (mainly Glc, Fru, and Suc) were eluted with 15 mL of water through both columns. Amino acids were eluted with 20 mL of 2 N HCl from the H^+ column and organic acids with 20 mL of 6 N formic acid from the Cl^- column. Each of these three fractions was then kept at −80°C, freeze-dried, and solubilized in 200 μL of water before isotopic analysis. The dried ethanol-insoluble fraction of the sample was extracted twice with 20 mL of water at 70°C. After centrifugation at 4000*g* for 10 min, the combined soluble extract corresponding to phosphate sugars was freeze-dried, dissolved in 1 mL of water, and used for MS analysis. The residue was treated with a protease from *Streptomyces griseus* (type IV, Sigma) at 30°C for 24 h in a 100-mM Na phosphate buffer at pH 7.5. The supernatant, containing amino acids and peptides from proteins, was then used for ^{13}C and ^{15}N determinations. The resulting pellet (mainly cellulose, lignin, and starch) was dried at 70°C for 24 h. The dry residue was mixed twice in 3 mL of 9 N HCl and centrifuged at 3000*g* for 10 min at 4°C. The final supernatants were pooled and the starch precipitation was obtained by addition of 24 mL of absolute methanol (Deléens and Garnier-Dardart, 1977). After centrifugation at 3000*g* for 15 min at 4°C, the residue (starch) was dried at 70°C for 4 h and solubilized in 1 mL of water before isotope analysis. The resulting pellet (cellulose and other cell-wall compounds) was dried at 70°C for 48 h, and then weighed and analyzed by MS.

Calculations of N Flow

The calculations performed in this study from ^{15}N data are based on various assumptions, many of which are supported by other works and have been summarized previously (Ourry et al., 1994). The apparent change (dN/dt) in N content in a plant organ during regrowth is the difference between N inflow and outflow from this organ:

$$dN/dt = \text{N inflow} - \text{N outflow} = N_{t+dt} - N_t \quad (1)$$

where N_t and N_{t+dt} are the N contents at time t and $t+dt$, respectively.

Because plants were nodulated and had no inorganic N supply during regrowth, the increase in ^{14}N content was derived from $^{14}N_2$ fixation. Therefore, N inflow derived from N_2 fixation during dt can be calculated from ^{15}N dilution as:

$$\text{N inflow} = N_{t+dt} \times (1 - E_{t+dt}/E_t) = \text{N derived from fixation} \quad (2)$$

where E_t and E_{t+dt} are ^{15}N atom % excess in the organ N measured at time t and $t+dt$, respectively. N outflow from a plant organ, corresponding to endogenous N remobilization during dt, can therefore be calculated from Equation 1:

$$\text{N outflow} = N_t - N_{t+dt} + \text{N inflow} \quad (3)$$

Substitution from Equation 2 gives:

$$\text{N outflow} = (N_t \times E_t - N_{t+dt} \times E_{t+dt})/E_t \quad (4)$$

Natural ^{15}N abundance ($A = 0.3663\%$) of atmospheric N_2 was used as a reference for ^{15}N analysis.

RESULTS

Dry-Matter Production and Relative Distribution of ^{15}N and ^{13}C within the Plant

The only significant dry matter production following defoliation was obtained from regrowing tissues (Fig. 1A), with the maximum growth rates occurring 10 d after shoot removal. The leaf-to-stem dry weight ratio declined during regrowth from 3.9 at 10 d to 1.3 at 30 d after defoliation. The dry weight of lateral roots and taproots decreased during the first 3 and 10 d, respectively, and then increased again to values close to those of the day of defoliation (Fig. 1B). The mean value of the ^{13}C excess found in whole plants and lost through respiration for all harvested plants ($n = 18$) was, at any time, 353 ± 36 μg ^{13}C plant^{-1}. This low variability in the ^{13}C recovered at each harvest, resulting mostly from biological variation, shows that no significant leaks of $^{13}CO_2$ occurred in the labeling system and, moreover, that the CO_2 trapping systems used in this experiment were quantitatively valid. Similarly, the total amount of ^{15}N remained constant at 165 ± 7 μg ^{15}N plant^{-1} for all harvested plants ($n = 18$), and the nutrient solution analysis detected no trace of ^{15}N during the chase period, suggesting that there was no significant efflux of ^{15}N into the nutrient solution.

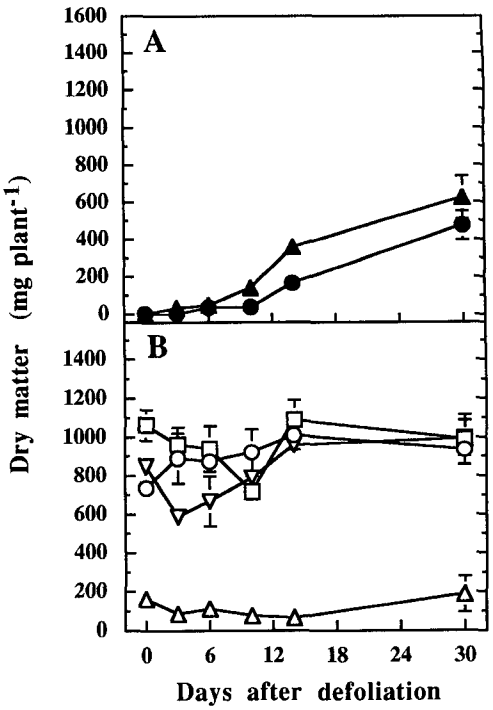

Figure 1. Changes in dry matter (mg plant^{-1}) of regrowing tissues (A) (▲, regrowing leaves; ●, regrowing stems) and remaining organs (B) (△, crown leaves; ○, crown stems; □, taproot; ▽, lateral roots) following shoot removal in alfalfa. Vertical bars indicate ± SE ($n = 3$) when larger than the symbol.

Since there was no significant loss of isotopes, the relative distribution of ^{13}C and ^{15}N excess within the plant could be calculated (Fig. 2). The decrease in ^{13}C excess relative distribution (Fig. 2A) in organs remaining after defoliation (lateral roots, taproots, crown stems, and crown leaves) was greater than that for ^{15}N excess (Fig. 2B). About 56% of the initial ^{13}C excess content of these organs was lost during the first 14 d of regrowth, and more than 73% was lost after 30 d (Fig. 2A). Most was lost by root respiration (83% of total ^{13}C excess lost from remaining organs during 30 d), whereas a smaller proportion (about 13% of initial ^{13}C content) was mobilized and translocated to the regrowing shoot, where 63% was then lost to foliar respiration. The final ^{13}C excess content of regrowing leaves and stems represented less than 5% of the labeled C found in the plant on the day of defoliation.

When ^{13}C changes were considered in absolute terms (Fig. 3) and related to loss of C by respiration (Fig. 3A), similar amounts of CO_2 were apparently generated by foliar and root respiration, although different substrates were used. These were derived mostly from nonlabeled C in the regrowing shoot (Fig. 3B) and were therefore linked mainly to photosynthetic activity, rather than from labeled compounds mobilized from the roots and translocated to the shoot. About 68% of the total respiratory loss of ^{13}C from the root system during 30 d of regrowth occurred during the first 2 weeks. Due to the experimental procedure used, which allowed spatial separation of roots and aerial tissue compartments, it is difficult to determine

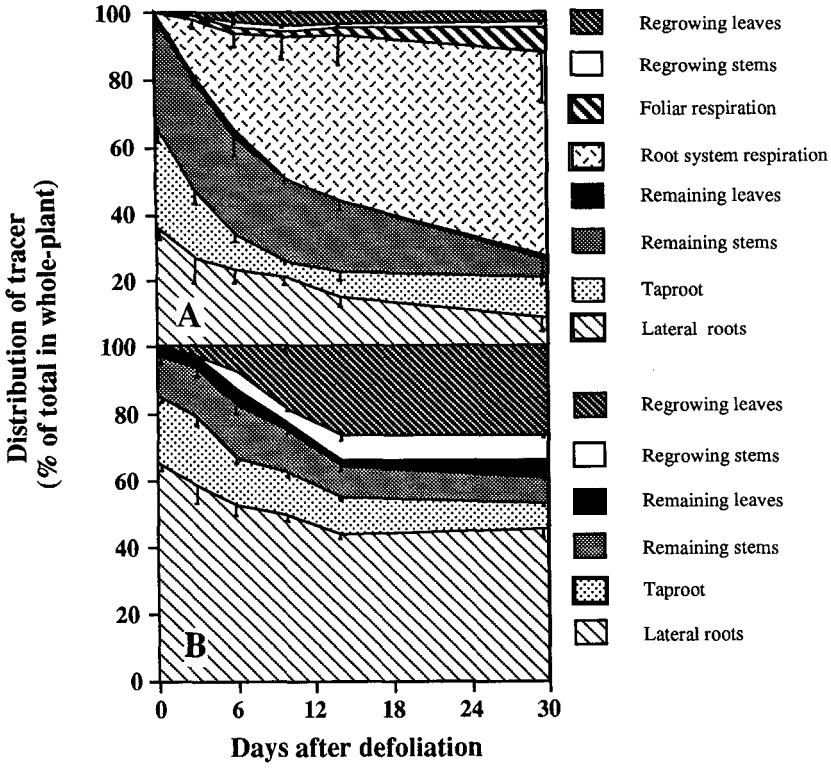

Figure 2. Relative distribution (percentage of total isotope) of ^{13}C excess in the different tissues and lost by respiration (A) and ^{15}N excess in individual organs during regrowth after defoliation (B) of alfalfa. Vertical bars indicate SE for $n = 3$.

whether the ^{13}C lost by shoot respiration was linked to the translocation of labeled compounds used for the respiratory process by regrowing tissues or whether this labeled C was lost by respiratory activity of the crown (leaves and stems remaining after defoliation). The increases in ^{13}C excess contents in regrowing leaves and stems at d 6 (Fig. 3C), and the following slight decreases at d 10, however, suggest that some labeled compounds were first mobilized and translocated to the shoot and subsequently used as substrates for respiration. The amount of ^{13}C in the leaves of the crown did not change significantly during regrowth, whereas the labeled C content of taproots, lateral roots, and stems of the crown decreased by 72% within 30 d (Fig. 3D). This process was faster in taproots and occurred mainly during the first 10 d.

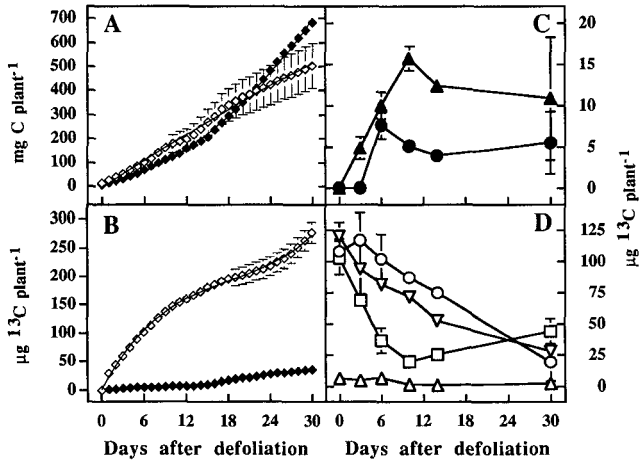

Figure 3. Time course of cumulative C (A) and ^{13}C losses (B) by foliar (♦) or root respiration (◇), and changes in ^{13}C excess content of regrowing tissues (C) and remaining organs (D) during regrowth of alfalfa following shoot removal. Regrowing leaves (▲) and stems (●), crown leaves (△) and stems (○), taproot (□), and lateral roots (▽). Vertical bars indicate ± SE ($n = 3$) when larger than the symbol, except for foliar respiration, for which values are the mean of triplicate measurements of shoot atmosphere.

Although the apparent decline in ^{15}N content (Fig. 2B) in the organs remaining after defoliation (−34% in 30 d) was smaller than the decline in ^{13}C, most of the remobilized ^{15}N was recovered in regrowing leaves and stems (Fig. 2B). The main source organs for mobilization of ^{15}N were lateral roots and taproots, whereas regrowing leaves provided a stronger sink than did stems. Essentially, all net ^{15}N translocation was complete within 14 d, in contrast to C, which was used throughout regrowth.

A comparison of the ^{15}N and ^{13}C content in alfalfa organs on the day of defoliation and 30 d later after shoot removal or without previous defoliation (control plants) is given in Table I. The ^{15}N content in leaves, crown stems, and lateral roots of nondefoliated plants did not change significantly after 30 d of growth. A marked decrease in ^{15}N was observed in the stems of control plants after 30 d, although a large increase in N labeling was detected in the crown leaves and taproot (Table I). The ^{15}N content of the taproot of nondefoliated alfalfa represented approximately 14% of the total ^{15}N in the nondefoliated plant, compared with only 6% on the day of defoliation (Table I), showing an accumulation of N in this organ. An extensive mobilization of ^{15}N and ^{13}C was observed (Table I; Fig. 3D) after 30 d of

Table I. *Comparison of isotope contents in the different organs of alfalfa on the day of shoot removal (d 0) and 30 d later (d 30) in regrowing or nondefoliated plants*

Values are given as the mean ± SE for $n = 3$.

Organ	d 0		d 30 (after cutting)		d 30 (uncut)	
	^{15}N excess	^{13}C excess	^{15}N excess	^{13}C excess	^{15}N excess	^{13}C excess
			μg plant^{-1}			
Leaves	325 ± 18	624 ± 89	43 ± 2	11 ± 8	301 ± 15	498 ± 23
Stems	84 ± 20	869 ± 71	13 ± 1	6 ± 4	55 ± 6	476 ± 35
Crown leaves	5 ± 1	6 ± 3	9 ± 3	2 ± 1	33 ± 3	3 ± 1
Crown stems	20 ± 3	108 ± 10	12 ± 1	19 ± 8	14 ± 2	22 ± 3
Taproots	33 ± 2	103 ± 13	13 ± 2	44 ± 2	88 ± 9	113 ± 21
Lateral roots	107 ± 3	120 ± 11	74 ± 5	28 ± 5	109 ± 4	77 ± 16
Total	574 ± 46	1830 ± 197	164 ± 13	110 ± 28	600 ± 40	1189 ± 100

regrowth in taproots and lateral roots of defoliated compared with nondefoliated plants.

In nondefoliated plants, the amount of ^{13}C in leaves and stems declined by 20 and 45%, respectively, between 0 and 30 d of growth (Table I). A very low increase in ^{13}C content was observed in the taproots (+10%) of nondefoliated plants over the same period. Comparison of the total amount of ^{13}C in alfalfa on the day of defoliation and 30 d later in nondefoliated plants showed that approximately 35% of the initial ^{13}C content was used for respiration processes. The amount of ^{13}C decreased significantly in the aerial organs and lateral roots of uncut plants after 30 d of growth. Only the taproot seemed to maintain its ^{13}C content in nondefoliated plants.

Origin of Regrowing Shoot N

Considering these data, and using Equations 2 and 4, the origin of shoot N was separated into that from storage compounds in the remaining organs and that derived from N_2 fixation, which was subsequently translocated to the shoot (Fig. 4). The N dilution process in regrowing alfalfa herbage has been described previously (Salette and Lemaire, 1981; Lemaire et al., 1992) and was also found in this experiment. Probably due to the decrease in the leaf-to-stem ratio with growth, and also to progressive senescence of the leaves, total N concentration in the shoot decreased from 70 mg N g^{-1} dry matter to less than 40 mg N g^{-1} dry matter, for a shoot dry weight increase of 1.4 g dry matter plant^{-1}. In the meantime, the contribution of N mobilization from reserves to the shoot decreased more strongly, accounting for about 90% of total shoot N at the beginning (d 3) to approximately 50% after 30 d (Fig. 4, inset). The contribution of N derived from N_2 fixation and further translocated to the shoot progressively increased with shoot growth.

Distribution of ^{15}N and ^{13}C within Different Biochemical Pools

The ^{15}N remobilized from the organs remaining after shoot removal was used mostly during regrowth for protein synthesis (Fig. 5) in regrowing leaves and stems and, to a considerably lesser extent, it accumulated in amino acids. Nearly equal amounts of ^{15}N were mobilized from labeled proteins in taproots and lateral roots (approximately 20 μg), but this represented a much larger proportion of labeled N stored in taproots (66%) than in lateral roots (30%) (Fig. 5). Amino acid ^{15}N was also largely mobilized, e.g. in taproots, where labeled N content increased slightly during the first 3 d and then decreased after 30 d by about 83% of the highest ^{15}N content found at d 3. Similar values were found for the amino acids in lateral roots, although their ^{15}N content was low compared with that of proteins (Fig. 5).

In crown stems, crown leaves, taproots, and lateral roots, the initial ^{13}C content of soluble sugar and starch fractions declined very quickly during the first 10 d to reach nearly a 0 value after 14 d of regrowth (Fig. 6). More surprisingly, a decrease in ^{13}C content was also found in the cellulose and cell-wall compounds of crown leaves, crown stems, and lateral roots, but remained unchanged in the taproot,

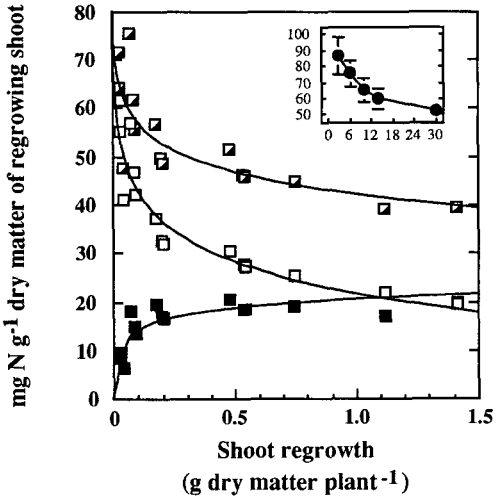

Figure 4. Changes in the origin of shoot N as a function of growth after defoliation of alfalfa. Origin of shoot N as N remobilized (□) from reserves of crowns and root system, exogenous N (■) derived from N_2 fixation, and total N (N remobilized + exogenous N, ◪) in shoots. Inset, Partitioning of N reserves expressed as the proportion of total N in regrowing tissues. Vertical bars indicate ± SE for $n = 3$. Equation curves were: N% = 2.29 dry matter$^{-0.257}$, N% = 2.14 dry matter$^{-0.208}$, and N% = 4.23 dry matter$^{-0.124}$ for N remobilized, exogenous N, and (N remobilized + exogenous N), respectively.

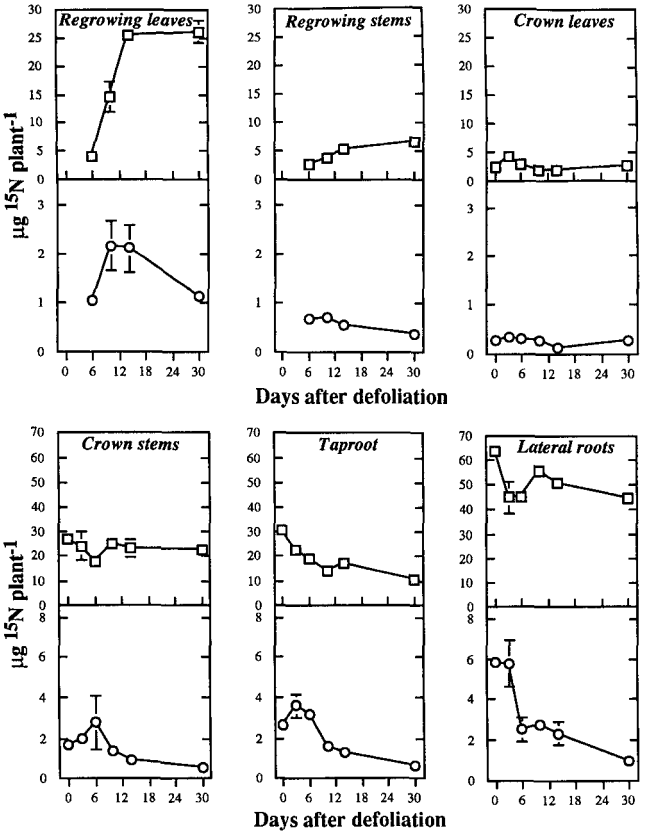

Figure 5. Changes in ^{15}N excess content in different N fractions of regrowing tissues and remaining organs during regrowth after defoliation of alfalfa. Amino acids (○) and proteins (□). Vertical bars indicate ± SE (n = 3) when larger than the symbol.

indicating that the cell-wall compound fraction had a low apparent turnover compared with starch and other fractions. This was supported by the pattern of ^{13}C incorporation in the same chemical fraction of regrowing leaves and stems during the first 10 d and the small decrease in the labeled C content during the following 20 d. All other fractions (starch, proteins, amino acids, organic acids, phosphate sugars, and soluble sugars) were nearly depleted in ^{13}C within 10 d of regrowth. The contribution of each of these fractions to the mobilization of ^{13}C toward translocation to shoot or root respiration was proportional to their C content (data not shown), and subsequently to their ^{13}C content: cell-wall compounds and cellulose > starch ≥ soluble sugars ≥ proteins > organic acids ≥ amino acids ≥ phosphate sugars. In regrowing tissue, at the end of regrowth the quantitative incorporation of labeled C occurred in a different order: cell-wall compounds and cellulose > starch ≥ proteins > organic acids > amino acids > phosphate sugars ≥ soluble sugars.

Origin of Regrowing Shoot C

The above data were used to determine the flow of ^{13}C and ^{15}N from the N pools (amino acids and proteins) of organs remaining after shoot removal (Table II) between the day of defoliation and 30 d later. Results show that both isotopes were similarly mobilized from a specific N pool. Between 68 and 90% (Table II) of initial ^{15}N or ^{13}C contents was lost from the amino acid pool (Table II). From a strictly quantitative point of view, proteins provided a source of ^{15}N that was 4- to 10-fold greater than that of amino acids (see, for example, lateral roots and taproots in Table II). A total efflux of about 53 µg ^{15}N plant^{-1} and 9.6 µg ^{13}C plant^{-1} occurred from N pools in organs remaining after shoot removal during the 30 d of regrowth.

If the relative contribution from the N pools of remaining organs to regrowing shoot ^{13}C have to be estimated, then several assumptions giving different and voluntarily extreme results need to be considered. Depending on the ^{15}N and ^{13}C redistribution to regrowing tissues, the first two propositions are either that ^{15}N and ^{13}C are remobilized in a similar manner (assumption 1) or are not remobilized (assumption 2). These propositions were suggested by Kim et al. (1993a), who showed that ^{15}N was translocated to the shoot through xylem vessels in mainly amide form (about 70% of amino acids in the xylem sap being Asn and Gln). The synthesis of amides by deamination involved the production of organic acids that could either be mobilized (assumption 1) or not be mobilized (assumption 2) to the regrowing shoot. We also assumed that the ^{13}C lost as CO_2 from the shoot could come either mainly from crown res-

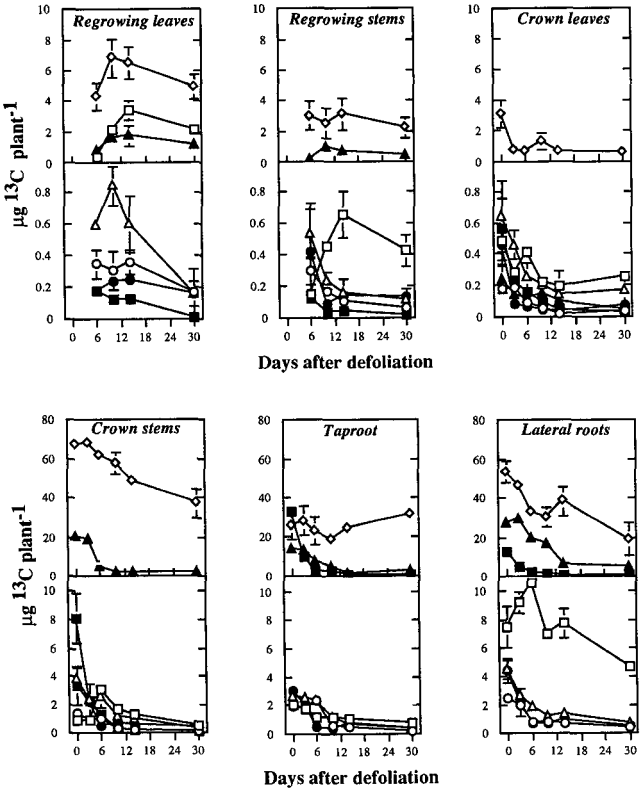

Figure 6. Changes in ^{13}C excess content in different fractions of regrowing tissues and remaining organs during regrowth after defoliation of alfalfa. Cell-wall compounds and cellulose (◇), starch (▲), organic acids (△), proteins (□), amino acids (○), phosphate sugars (●), and soluble sugars (■). Vertical bars indicate ± SE (n = 3) when larger than the symbol.

Table II. *Amounts of ^{15}N or ^{13}C excess (μg plant^{-1}) found on the day of shoot removal (d 0) and after 30 d of regrowth (d 30), expressed as N-fractions of remaining organs, dry weight of regrowing tissues, and foliar respiration*

Values are given as the mean ± SE for $n = 3$.

Organ	N Fraction	Isotopic Excess Content at d 0		Isotopic Excess Content at d 30		Balance d 0–d 30		Loss of Labeling as Percentage of Initial Content	
		^{15}N	^{13}C	^{15}N	^{13}C	^{15}N	^{13}C	^{15}N	^{13}C
		μg isotope plant^{-1}						%	
Lateral roots	Amino acids	5.8 ± 0.2	2.4 ± 0.15	1 ± 0.3	0.4 ± 0.1	4.8	2.05	83	84
	Proteins	63.9 ± 1.7	7.5 ± 1.4	44.1 ± 2.5	4.6 ± 0.3	19.1	2.8	30	38
Taproots	Amino acids	2.6 ± 0.02	1.9 ± 0.2	0.6 ± 0.2	0.2 ± 0.1	2.1	1.8	78	90
	Proteins	30.6 ± 1.4	2 ± 0.2	10.5 ± 0.5	0.7 ± 0.3	20.1	1.3	66	64
Crown leaves	Amino acids	2.6 ± 0.02	0.2 ± 0.04	0.6 ± 0.01	0.03 ± 0.02	2.1	0.15	78	82
	Proteins	2.3 ± 0.3	0.5 ± 0.05	2.6 ± 0.7	0.2 ± 0.03	ns[a]	0.2	ns	48
Crown stems	Amino acids	1.7 ± 0.3	1.3 ± 0.3	0.5 ± 0.1	0.1 ± 0.02	1.1	1.1	68	90
	Proteins	27 ± 2.9	0.9 ± 0.1	22.7 ± 0.1	0.7 ± 0.1	4.3	0.2	16	18
	Total	136.6	16.7	83.3	7.1	53.5	9.6	39	51
Regrowing leaves	Total dry weight			43.6 ± 1.5	10.9 ± 7.5	43.6	10.9		
Regrowing stems	Total dry weight			12.7 ± 0.8	5.51 ± 3.8	12.7	5.5		
Foliar respiration	CO_2				27.6		27.6		
	Total					56.3	44.1		

[a] ns, Not significant.

piration (assumption 3) or from regrowing tissue respiration (assumption 4). Thus, depending on whether these assumptions are considered alone or together, the amount of ^{13}C from N pools in remaining organs (9.6 μg ^{13}C plant^{-1}) may range from 58% (assumptions 1 and 3), to 35% (assumptions 2 and 3), to 22% (assumptions 1 and 4), to 14.5% (assumptions 2 and 4) of ^{13}C recovered in regrowing leaves and stems or lost by regrowing tissue respiration. Although this range is broad, it should be kept in mind that only a small proportion of ^{13}C, and thus of C, was mobilized from storage organs to regrowing shoot (Fig. 2A). These estimations are supported by the relative ^{13}C distribution in proteins and amino acids of stems and leaves (Fig. 6), accounting for 32 and 18%, respectively, of the total labeled C contents of these tissues at d 14. Therefore, most regrowing shoot C was derived directly from the photosynthetic activity of regrowing leaves. Finally, it can be suggested that a significant proportion of the small amount of C mobilized to regrowing shoot (between 14.5 and 58%) was associated with N translocation.

DISCUSSION

Previous results obtained with alfalfa suggested that regrowth following defoliation was not related to carbohydrate availability in the roots and crown, but mainly to prior storage of N-containing compounds (Ourry et al., 1994). In contrast, experiments using ^{14}C pulse-chase labeling during regrowth showed that significant amounts of labeled compounds were translocated to regrowing tissues (Hodgkinson, 1969; Ta et al., 1990). Given that (a) $^{14}CO_2$ feeding was performed for a short period (1 d) and therefore did not allow a uniform labeling of storage compounds, (b) respiratory losses of ^{14}C were not directly quantified, (c) label distribution was often expressed as relative specific activity, and (d) the origin of ^{14}C compounds from the source organs was not determined, we conclude that the contribution of stored C in the synthesis of new tissues has been overestimated. A full balance of ^{13}C flow after a 10-d labeling period allowed a relative quantitative assessment of the use of stored C in different processes.

The results obtained by comparison of plants harvested on the day of defoliation with regrowing or growing nondefoliated plants harvested 30 d later (Table I) show that shoot removal produced considerable depletion of ^{13}C and ^{15}N in taproots, lateral roots, and crown stems after 30 d of regrowth. These observations demonstrate that the mobilization of C and N reserves from source organs to support early regrowth and plant metabolism is large, and that the isotope depletion observed after shoot removal was not due to a normal turnover process, as suggested by data from nondefoliated plants (Table I). There is a relatively large decline of ^{13}C content in the cell-wall compounds of crown stems, crown leaves, and lateral roots (Fig. 6). Such a decrease of labeled C in cell-wall compounds had already been suggested in studies of other forage species during regrowth following shoot removal. For example, the net loss of nonstructural carbohydrates from the root system of *Dactylis glomerata* (Davidson and Milthorpe, 1966) after moderate or severe defoliation could account for only a fraction of the total respiratory C loss from the roots (Richards, 1993). Mobilization of substrates other than nonstructural carbohydrates, perhaps including hemicellulose, proteins, and organic acids, must have been required for root respiratory activity (Davidson and Milthorpe, 1966). Other studies have suggested the importance of these alternative substrates after severe defoliation (Chung and Trlica, 1980;

Dewald and Sims, 1981; Richards and Caldwell, 1985). It should be pointed out that in the present study, parts of the crown tissues were largely subjected to irreversible senescence, leading to leaf and stem death, which might explain the loss of ^{13}C from cell-wall compounds. A similar process for lateral roots remained difficult to explain under our conditions, and senescence was not observed.

Most labeled C was used for root system tissue respiration (82% of the ^{13}C lost; Fig. 2A), showing that the maintenance costs of organs remaining after defoliation were very high and therefore represented a major expenditure of energy. Since N_2 fixation was strongly repressed after defoliation (Ta et al., 1990; Kim et al., 1993b), leading to decreased respired CO_2 production by nodules (Denison et al., 1992), respiration by underground parts resulted mainly from root physiological processes. It was shown in nodulated soybean (*Glycine max* [L.] Merr.) that nodule respiration averaged more than 50% of the total respiration of underground parts (Kouchi et al., 1986; Hansen et al., 1992). When N_2 fixation was reduced, for example by adding nitrate to the nutrient solution, the respiration rates of nodules decreased strongly in alfalfa (Arrese-Igor et al., 1992) and soybean (Hansen et al., 1992), whereas root CO_2 production was stimulated (Hansen et al., 1992). Similarly, Denison et al. (1992) showed that detopping decreased the production of CO_2 by alfalfa nodules by 65%. It is now acknowledged that different substrates are used for root and nodule respiration: stored compounds in roots and recently fixed C in nodules. Because we do not know which physiological processes in roots require large amounts of C, it is difficult to predict whether a reduction in C storage will be detrimental to the long-term regrowth of plants. Detrimental effects were not found in plants in which the root initial starch content varied from 57 mg to less than 7 mg plant^{-1} (Ourry et al., 1994), since regrowth yield was higher in the latter plants. We have shown that substrates used for respiration in regrowing tissue were derived mainly from newly fixed C (Fig. 3, A and B). Moreover, the contribution of labeled C of roots and crown to the synthesis of new tissues was very low (less than 5% of total ^{13}C was recovered in shoot), between 14.5 and 58% of this being associated with mobilization and further translocation of N-containing molecules (Figs. 5 and 6; Table II).

Although the apparent decreases of labeled N content in roots and crown (Fig. 2B) were small during regrowth compared with ^{13}C (Fig. 2A), most of the ^{15}N was recovered in the regrowing shoot (34% of total ^{15}N after 30 d of regrowth). Our results are a little higher than those of Ta et al. (1990), who concluded that about 25% of total root N was translocated into regrowing shoots 28 d following harvest of 8-week-old alfalfa plants. This suggests that stored N contributes substantially (Fig. 4) to the synthesis of amino acids and proteins in the leaves and stems (Fig. 5).

The consequences of low storage of N compounds before shoot removal have been shown in alfalfa (Ourry et al., 1994) and in *Lolium temulentum* L. (Ourry et al., 1996). A shortage of N reserves results in reduced shoot yield in both species, but the forage legume seems to be more affected. Such a difference between fixing and nonfixing forage species may be explained by the higher plasticity of mineral N uptake as nitrate or ammonium by roots of the latter, which is able to compensate by an increase in uptake rate, whereas a compensatory increase of N_2 fixation and/or inorganic N uptake rates may not be possible in the fixing forage species.

Further work is needed to evaluate the causal relationships of modulated storage of C and N and their respective consequences on root maintenance processes and shoot yield. The role of storage compounds during regrowth may have several implications during spring growth and postharvest regrowth, as well as in comprehensive approaches to understanding the factors of intra- and interspecific competition that govern forage persistence and productivity.

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